Identifying Regions of Membrane Proteins in Contact with Phospholipid Head Groups: Covalent Attachment of a New Class of Aldehyde Lipid Labels to Cytochrome *c* Oxidase†

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ABSTRACT: A series of amine-specific reagents based on the benzaldehyde reactive group have been synthesized, characterized, and used to study beef heart cytochrome *c* oxidase reconstituted in phospholipid bilayers. The series contained three classes of reagents: lipid-soluble phosphodiesters having a single hydrocarbon chain, phospholipid analogues, and a water-soluble benzaldehyde. All reagents were either radiolabeled or spin-labeled or both. The Schiff bases formed by these benzaldehydes with amines were found to be reversible until the addition of the reducing agent sodium cyanoborohydride, whereas attachment of lipid-derived aliphatic aldehydes was not readily reversible in the absence of the reducing agent. The benzaldehyde group provides a convenient method of controlling and delaying permanent attachment to integral membrane proteins until after the reconstitution steps. This ensures that the lipid analogues are located properly to identify amine groups at the lipid–protein interface rather than reacting indiscriminately with amines of the hydrophilic domains of the protein. The benzaldehyde lipid labels attach to cytochrome *c* oxidase with high efficiency. Typically, 20% of the amount of lipid label present was covalently attached to the protein, and the number of moles of label incorporated per mole of protein ranged from 1 to 6, depending on the molar ratios of label, lipid, and protein. The efficiency of labeling by the water-soluble benzaldehyde was much less than that observed for any of the lipid labels because of dilution effects, but equivalent levels of incorporation were achieved by increasing the label concentration. Electron spin resonance spectra of a nitroxide-containing phospholipid analogue covalently attached to reconstituted cytochrome *c* oxidase exhibited a large motion-restricted component, which is characteristic of spin-labeled lipids in contact with the hydrophobic surfaces of membrane proteins. The line shape and splittings were similar for covalently attached label and label free to diffuse and contact the protein molecules in the bilayer, providing independent evidence that the coupling occurs at the protein–lipid interface. The distribution of the benzaldehyde reagents attached to the polypeptide components of cytochrome *c* oxidase was examined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The labeling pattern observed for the lipid analogues was not affected by the presence of the nitroxide moiety on the acyl chains but was dependent on the molar ratio of labeling reagent to protein. With the lipid labels, band VII was the most heavily labeled, and significant labeling of bands III, V, and VI was observed at higher labeling ratios. There was little or no labeling of bands I, II, and IV. A different labeling pattern was observed with the water-soluble label, providing additional evidence that the lipid-like benzaldehyde reagents react with cytochrome *c* oxidase from the confines of the bilayer. Thus, these new labels have the necessary specificity and reactivity to be useful in correlating sequence data with the structure and function of integral membrane proteins, particularly in identifying regions in contact with phospholipid head groups at the lamellar interface.

Cytochrome *c* oxidase functions as the final stage of cell respiration where electrons originating from oxidized foodstuffs are transferred from cytochrome *c* to molecular oxygen. The energy released in this process is conserved as a proton gradient across the membrane. The problem of resolving the structure of this complex membrane protein is gradually yielding to a combination of biochemical and biophysical techniques [for reviews, see Buse (1984), Capaldi et al. (1983), and Wikstrom et al. (1981)]. The enzyme contains two hemes and two copper atoms. SDS–PAGE[1] separates mammalian cytochrome *c* oxidases into seven major bands (I–VII), and some bands (V–VII) can be further resolved. The amino acid sequences of major polypeptides from yeast and mammalian cytochrome *c* oxidases have been determined. There are as many as 12–13 different polypeptides present in significant quantities, depending on the operational definition of what constitutes an intact enzyme complex (Buse, 1984; Kadenbach, 1983a,b). In counting the polypeptides and defining what constitutes a subunit, there remain possible complications from the presence of copurified components from other membrane proteins, polypeptide heterogeneity, and modification of cytochrome *c* oxidase during the isolation procedures. A low-resolution three-dimensional structure obtained from two-dimensional crystals shows that beef heart cytochrome *c* oxidase is a Y-shaped protein with two domains (M_1 and M_2) exposed to the matrix side of the inner membrane and one larger domain (C)

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[1] Abbreviations: DOPC, 1,2-dioleoyl-3-*sn*-phosphatidylcholine; ESR, electron spin resonance; proxyl, 2,2-dialkyl-5,5-dimethylpyrrolidine-*N*-oxyl; CDI, carbonyldiimidazole; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SPE, 0.25 M sucrose, 50 mM phosphate, and 1 mM ethylenediaminetetraacetic acid; cm, complex multiplet; bm, broad multiplet; d, doublet; t, triplet; s, singlet.

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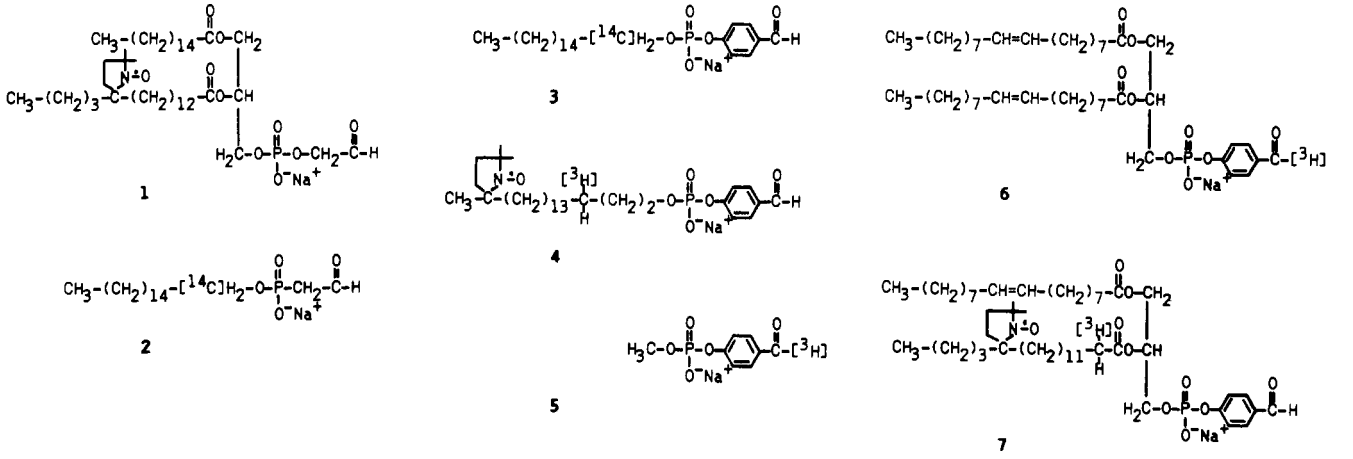

FIGURE 1: Structures of aldehyde lipid labels used in this study. Molecules **1** and **2** contain a phosphate acetaldehyde and a phosphonate acetaldehyde group, respectively. In molecules **3**–**7**, a benzaldehyde is the reactive functional group. Molecule **5** is a water-soluble benzaldehyde used for comparison with the long-chain membrane-soluble molecules **3**, **4**, **6**, and **7**.

extending out into the aqueous layer on the cytoplasmic side (Fuller et al., 1979). Three-dimensional crystals have been obtained (Ozawa et al., 1980), but so far no high-resolution electron density maps have been reported.

To supplement efforts to obtain high-resolution three-dimensional structures of this and other complex membrane proteins, a fruitful approach has been chemical modification, with the long range goal of determining the location of specific amino acids in relation to subunit structure and the phospholipid bilayer. Several water-soluble labeling reagents such as DABS (diazonium benzenesulfonate), NAP-taurine [2-[*N*-(4-azido-2-nitrophenyl)amino]ethanesulfonate], and *N*-ethylmaleimide, iodination with lactoperoxidase, as well as antibody binding experiments have been used for surface mapping of cytochrome *c* oxidase [see Wikstrom et al. (1981)]. However, there are very few suitable lipid-soluble reagents for chemical labeling of membrane proteins. Such labels are needed, for example, in determining points at which the subunits of cytochrome *c* oxidase enter the phospholipid bilayer and in examining the role of phospholipids near the cytochrome *c* binding sites. One class of lipid-like labels that have been reported are the photoactivated arylazido phospholipids (Chakrabarti & Khorana, 1975; Bisson et al., 1979; Griffith & Jost, 1979; Prochaska et al., 1980). The nitrene group of these labels most likely reacts with a variety of protein functional groups through mechanisms that are as yet poorly understood. Thus, they are useful as general protein modifying reagents but not for labeling of a specific class of amino acids. In the present study we have prepared and characterized a series of radiolabeled and spin-labeled aldehyde-containing lipid derivatives designed for membrane biochemical studies (Figure 1). Aldehydes were selected because of their known specificity for reaction with amino groups. Furthermore, the reaction can be controlled by selecting the time at which the Schiff base is reduced; i.e., the formation of an irreversible covalent bond can be delayed until membrane reconstitution is complete and the reactive lipid analogue is inserted correctly into the bilayer. By combining ESR spectroscopic measurements with analysis of labeling patterns, information is obtained on the reactive behavior of these aldehyde probes with amino groups of cytochrome *c* oxidase in contact with the phospholipid head groups of the lipid bilayer.

Experimental Procedures

Syntheses of Aldehyde Labels. The syntheses of **1** and **2** are given in the supplementary material (see paragraph at end of paper regarding supplementary material). The syntheses

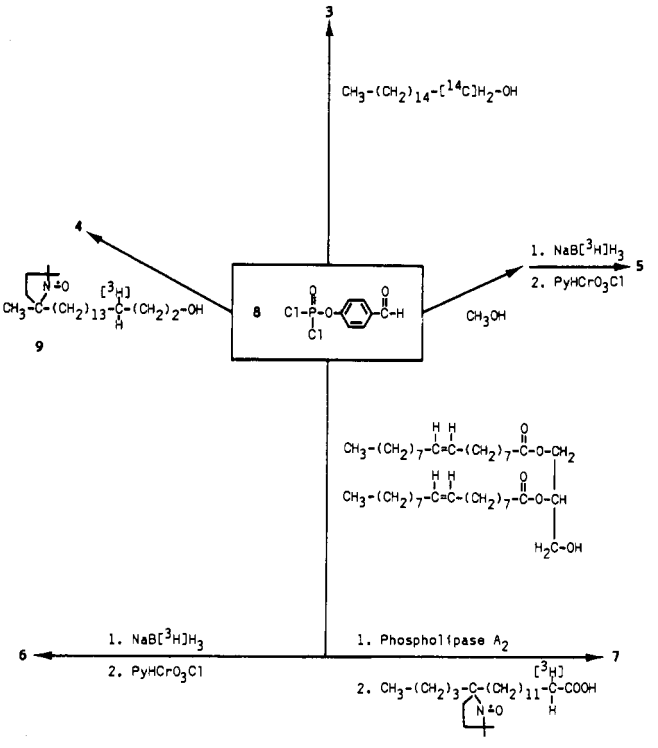

FIGURE 2: Overview of the synthetic routes followed to obtain the benzaldehyde-containing lipid labels **3**–**7**.

of the benzaldehyde labels **3**–**7**, which are more central to this work, are outlined in Figure 2 and described in detail below.

4-Formylphenyl Phosphorodichloridate (**8**). To a solution of 4-hydroxybenzaldehyde (2.442 g, freshly sublimed) in boiling water (14 mL), 4.2 mL of 10 N NaOH was added and the resulting faintly yellow solution cooled rapidly in an ice bath. The white crystals of the sodium salt were collected, washed with chilled water, and dried under vacuum at 100 °C overnight to give sodium 4-formylphenolate (2.042 g, 71%) as a white powder. A 3-g sample of dry sodium 4-formylphenolate was added to freshly distilled phosphorus oxychloride (20 mL) at 0 °C with vigorous stirring under nitrogen. The resulting yellow suspension was centrifuged. The clear supernatant was diluted with dry toluene (30 mL) and concentrated under vacuum to about 8 mL of red oil. This was diluted with toluene (20 mL) and concentrated to remove excess phosphorus oxychloride. After a final evaporation from toluene, the resulting greenish oil was extracted with dry carbon tetrachloride (30 mL) and rapidly passed through a

silica gel column, fresh carbon tetrachloride being used as a rinse. The combined eluents were concentrated, giving a faint red heavy oil (2.46 g, 49%). Prior to use, aliquots of this oil were freshly purified by flash distillation (0.005 mmHg, bath temperature 135 °C) in a sublimator apparatus. For example, a 1.18-g aliquot produced 0.70 g (59%) of pure 4-formylphenyl phosphorodichloridate (**8**) as a clear colorless oil, which solidified in the freezer: NMR ($CDCl_3$) δ 10.03 (s, 1 H), 7.98 (d, 2 H), 7.48 (d, 2 H).

Sodium n-Hexadecyl 4-Formylphenyl Phosphate (**3**). Dichloridate **8** (65 μL, 0.40 mmol) dissolved in dry ether (1 mL) at 0 °C was treated with a solution of hexadecanol (24 mg) in ether (2 mL) containing 2,6-lutidine (116 μL). After being stirred for 15 min at 0 °C and 15 min at 25 °C, the mixture was recooled to 0 °C and diluted with ether (4 mL) followed by water (0.2 mL). After 10 min the ether layer was washed with water and evaporated, and the residue was dissolved in chloroform (1 mL) and toluene (1.5 mL). The solvents were evaporated, and the process was repeated. The waxy residue was dissolved in 0.1 N NaOMe in methanol (1.5 mL) and concentrated to dryness. The residue was purified by preparative TLC over silica gel (developed in chloroform/methanol/water, 4:1:0.1). The band at R_f 0.2 was collected, giving the sodium salt, **3** (36 mg, 82%). The analytical sample was obtained from a second preparative TLC. Anal. Calcd for $C_{23}H_{38}O_5PNa \cdot {}^1/_3H_2O$: C, 60.78; H, 8.85. Found: C, 60.90; H, 8.83.

^{14}C-Labeled sodium hexadecyl 4-formylphenyl phosphate ([^{14}C]-**3**) was prepared similarly by starting with [1-^{14}C]-hexadecanol (ICN, 250 μCi, 55.9 mCi/mmol) diluted with 8.8 mg of hexadecanol. The final product obtained was 12.8 mg (70%) of [^{14}C]-**3** with a specific activity of 1.177×10^7 cpm/μmol.

17-Proxyl[3-^{3}H]octadecanol ([^{3}H]-**9**). To a suspension of N-chlorosuccinimide (66.6 mg) in dry toluene (1.5 mL) at 0 °C was added dropwise 6.94 mL of a 0.975 M solution of dimethyl sulfide in toluene. The mixture was cooled to −23 °C and treated dropwise with a solution of 15-proxylhexadecanol (113 mg; Keana & LaFleur, 1979) in toluene (0.5 mL). After 2 h at −23 °C, 0.53 mL of a 0.998 M solution of triethylamine in toluene was added. The mixture was allowed to warm to 23 °C, diluted with ether, washed with water, and then washed with brine. It was then dried (K_2CO_3) and concentrated to dryness, giving 15-proxylhexadecanal (86 mg, 75%): IR ($CHCl_3$) 1720 cm^{-1}. A total of 55 mg of this material in dry 2-propanol (0.5 mL) was added to an ampule containing sodium boro[^{3}H]hydride (Amersham, 9.8 Ci/mmol, 100 mCi), the mixture was stirred for 12 h, and cold $NaBH_4$ (6 mg) was added to assure complete conversion to the alcohol. After 2 h the mixture was cooled to 0 °C, water (0.1 mL) was added, and the solution was diluted with ether and washed with brine. The aqueous layer was back-extracted with fresh ether, and the combined ether phases were dried (K_2CO_3). Evaporation gave a yellow oil, which was purified by preparative TLC over silica gel. The yellow band amounted to 48 mg (87%) of 15-proxyl[1-^{3}H]hexadecanol. This was diluted with the corresponding cold alcohol (211 mg) and converted into 17-proxyl[3-^{3}H]stearic acid (158 mg) as previously described for the nonradioactive material (Keana & LaFleur, 1979).

To a solution of 17-proxylstearic acid (20.9 mg) in dry ether (1 mL) at 25 °C was added 1.0 mL of 2.0 M borane–dimethyl sulfide complex in THF (Aldrich). After 5 h, methanol (2 mL) was added and the mixture allowed to stand overnight at 5 °C. The solvent was removed and the residue purified by preparative TLC over silica gel (ether–pentane, 3:2). The yellow band at R_f 0.35 afforded 10 mg (49%) of 17-proxyloctadecanol (**9**) as a yellow waxy solid: MS, m/e 368 (M^+).

[^{3}H]-**9** was prepared similarly via reduction of 17-proxyl-[3-^{3}H]stearic acid (29.5 mg) with borane–dimethyl sulfide complex, except that the resulting oil and $Cu(OAc)_2 \cdot H_2O$ (3.0 mg) were dissolved in 95% ethanol (1.5 mL) containing 30% aqueous ammonium hydroxide (0.1 mL) and stirred under air for 14 h at 25 °C in order to reoxidize any reduced nitroxide groups. This precautionary step may not have been necessary because the proxyl moiety is resistant to reduction with borane–dimethyl sulfide complex (Keana et al., 1978). Preparative TLC gave 8.3 mg (28%) of the radioactive alcohol, [^{3}H]-**9**.

Sodium 4-Formylphenyl 17-Proxyl[3-^{3}H]octadecyl Phosphate (**4**). Cold **9** (10 mg) in dry ether (2 mL) containing 2,6-lutidine (90 μL) was added to 4-formylphenyl phosphorodichloridate (**8**) (50 μL) in ether (10 mL) at 0 °C, stirred for 15 min, and then stirred at 25 °C for 15 min. The reaction mixture was diluted with ether (4 mL) and washed with cold water. A total of 0.2 mL of 1 N NaOH was added and the mixture concentrated to dryness. The residue was purified by preparative TLC over silica gel (Whatman PK6F) with chloroform/methanol/water (4:1:0.1). The band at R_f 0.5 was collected, giving 6.8 mg (44%) of **4** as a pale yellow solid. Anal. Calcd for $C_{30}H_{50}NNaO_6P \cdot {}^1/_2H_2O$: C, 61.73; H, 8.81; N, 2.40. Found: C, 61.76; H, 8.61; N, 2.54. An analogous procedure using [^{3}H]-**9** (8.3 mg) afforded [^{3}H]-**4** (5.9 mg, 46%, sp act. 4.326×10^7 cpm/μmol).

Sodium rac-1,2-Dioleoylglycero-3-(4-[^{3}H]formylphenyl phosphate) ([^{3}H]-**6**). To an ice-cold stirred solution of freshly distilled **8** (104 mg) in dry ether (2 mL) was added dropwise under N_2 a solution of *rac*-1,2-diolein (50 mg; Sigma) and 0.1 mL of dry 2,6-lutidine in 1.8 mL of dry ether. The reaction mixture was stirred in an ice bath under N_2 for 15 min and then at room temperature for 75 min. After the reaction was shown to be complete by analytical TLC, the reaction mixture was diluted with 3 mL of ether and quenched with ice-cold distilled water (1.5 mL). The ether layer was washed with ice-cold distilled water (3 × 1 mL), ice-cold 0.1 N HCl (3 × 1 mL), and ice-cold distilled water (3 × 1 mL). All aqueous layers were combined and back-extracted with ether (3 × 4 mL). All ether layers were combined, and the solvent was evaporated, giving the acid form of **6** as a clear yellowish oil (yield 65 mg, 100%): NMR ($CDCl_3$) δ 0.86 (t, 6 H), 1.10–1.62 (cm), 1.98 (d, 8 H), 2.12–2.30 (bm, 4 H), 2.79 (bs, 1 H), 4.04–4.30 (bm, 4 H), 5.32 (t, 4 H), 7.83 (d, 2 H), 7.78 (d, 2 H), and 9.88 (s, 1 H).

To a stirred ice-cold suspension of **6** (65 mg) in dry ether (5 mL) was added ice-cold diazomethane (0.455 mmol) in ether (5 mL). The resulting clear yellow solution was stirred on ice under N_2 for 20 min and then allowed to warm up to room temperature. Excess diazomethane was removed with a stream of N_2, and concentration yielded the crude phosphotriester methyl-**6** which was purified by preparative TLC with 5% MeOH in $CHCl_3$ as the solvent. Repurification with the same method yielded analytically pure methyl-**6** (57.1 mg, 86%). Anal. Calcd for $C_{47}H_{79}O_9P$: C, 68.92; H, 9.72. Found: C, 68.64; H, 9.67. NMR ($CDCl_3$) showed the following: δ 0.87 (t, 6 H), 1.23–1.43 (bm), 1.58 (bt, 4 H), 2.00 (d, 8 H), 2.29 (t, 4 H), 3.88 (d, 3 H), 4.07–4.42 (cm, 4 H), 5.34 (t, 4 H), 7.37 (d, 2 H), 7.89 (d, 2 H), and 9.95 (s, 1 H).

Methyl-**6** (35 mg) was dissolved in 1 mL of dry 2-propanol (freshly distilled from CaH_2), and sodium borohydride (1 mg) was added. The resulting yellow suspension was stirred at room temperature under N_2 for 2 h. Ethylene glycol (0.081

mL, freshly distilled from anhydrous $MgSO_4$) was then added, and stirring was continued for 30 min. After evaporation of the solvent, the resulting yellow oil was extracted with ether (4 × 3 mL), and the combined ether extracts were concentrated to yield *rac*-1,2-dioleoylglycero-3-[methyl 4-(hydroxymethyl)phenyl phosphate] as a clear waxy oil. NMR ($CDCl_3$) indicated the complete absence of the aldehyde peak.

The crude alcohol (35.1 mg, dried azeotropically by repeated concentration from dry CH_2Cl_2) in 1 mL of dry CH_2Cl_2 was added to a solution of pyridinium chlorochromate (14.5 mg, dried for 24 h at 0.005 mmHg at 59 °C) in 0.35 mL of dry CH_2Cl_2. After being stirred for 3.5 h at room temperature under nitrogen, the reaction mixture was diluted with 10 mL of dry ether and filtered through a plug of anhydrous $MgSO_4$. The plug was washed with fresh ether (5 × 8 mL), and the combined ether layers were concentrated to yield a clear, pale yellow waxy oil. Preparative TLC with 4% MeOH in $CHCl_3$ as the solvent system gave pure triester methyl-**6** (yield 18.1 mg, 52%): NMR ($CDCl_3$) δ 0.88 (t, 6 H), 1.16–1.69 (cm), 1.99 (d, 8 H), 2.30 (t, 4 H), 3.88 (d, 3 H), 4.14–4.40 (bm cm, 4 H), 5.34 (t, 4 H), 7.36 (d, 2 H), 7.89 (d, 2 H), and 9.96 (s, 1 H).

Triester methyl-**6** (59.4 mg) was dried under high vacuum at 25 °C for 24 h and dissolved in methyl ethyl ketone (2.25 mL, freshly distilled from P_2O_5). Sodium iodide (14.2 mg, dried under high vacuum at the temperature of refluxing acetone) was added, and the resulting clear yellow solution was refluxed for 20 min. Concentration yielded crude sodium salt **6**, which was purified by preparative TLC with $CHCl_3$/MeOH/H_2O (65:25:4) as the solvent (yield 53.6 mg, 89%). Anal. Calcd for $C_{46}H_{76}O_9PNa \cdot 3H_2O$: C, 62.68; H, 9.38. Found: C, 62.84; H, 8.83.

[^{3}H]-**6** was prepared from methyl-**6** via reduction with sodium boro[^{3}H]hydride (100 Ci/mol, New England Nuclear), oxidation with pyridinium chlorochromate, and demethylation with NaI as described above. The final product (9.4 mg) had a specific radioactivity of 1.68×10^7 cpm/μmol and cochromatographed with cold **6**.

Sodium 1-Oleoyl-2-(14-proxyl[2-^{3}H]stearoyl)-sn-glycero-3-(4-formylphenyl phosphate) (**7**). To a solution of diacylbenzaldehyde (**6**) (43.6 mg) in 10 mL of ether (freshly distilled from $LiAlH_4$) were added 0.1 mL of borate buffer (0.1 M sodium borate, pH 7.8, containing 0.01 M $CaCl_2$) and 0.1 mL of the same buffer containing 1 mg/mL porcine pancreatic phospholipase A_2 (Nieuwenhuizen et al., 1974). Progress of the reaction was checked by HETLC (high-efficiency TLC plates, Analtech) with $CHCl_3$/MeOH/water (65:25:1) as the solvent. After a 20-min stir at room temperature, the reaction was complete (i.e., 50% hydrolysis due to the racemic nature of **6**), the solvent was evaporated and the residue dried under high vacuum for 2 h. The reaction mixture was dissolved in 1 mL of $CHCl_3$ containing 1% MeOH, filtered through a plug of cotton wool, and applied to a column (7 × 2 cm) of CM-cellulose (Whatman CM-52, sodium form). The column was eluted with 25-mL portions of 1, 5, and 10% MeOH in $CHCl_3$, 50 mL of 15% MeOH in $CHCl_3$, 25 mL of 20% MeOH in $CHCl_3$, 50 mL of 25% MeOH in $CHCl_3$, and 25-mL portions of 30 and 50% MeOH in $CHCl_3$, respectively. The pure *sn*-1 stereoisomer of **6** eluted at 15–20% MeOH in $CHCl_3$, whereas pure sodium 1-oleoyl-2-hydroxy-*sn*-glycero-3-(4-formylphenyl phosphate) eluted at 25–30% MeOH in $CHCl_3$. Concentration yielded 22.7 (100%) and 15.0 mg (100%) of the diacyl and monoacyl compounds, respectively.

In order to assess whether the reactive benzaldehyde group would remain intact under the conditions necessary for reacylating the monoacyl compound with tritiated 14-proxylstearic acid according to the procedure of Boss et al. (1975), a pilot reacylation was performed as follows: A solution of CDI (7.2 mg) in dry $CHCl_3$ (1 mL, freshly distilled from P_2O_5) was added to 12.6 mg of oleic acid (Sigma, dried under high vacuum). After 30 min at room temperature under dry nitrogen, this mixture was added to 8.0 mg of the monoacyl compound, and the resulting solution was kept at 55 °C under a gentle stream of dry nitrogen. At regular time intervals, the progress of the reaction was checked by HETLC with $CHCl_3$/MeOH/water (65:25:1) followed by ether/hexane/acetic acid (50:50:1) as the solvent systems. After the reaction was complete (3 h), the reaction mixture was taken up in $CHCl_3$ and quenched with 0.005 mL of distilled water. Removal of the solvent and drying under high vacuum gave a colorless waxy residue that was dissolved in 1 mL of $CHCl_3$ containing 1% MeOH and applied to the CM-cellulose column described above. The column was eluted with 25-mL portions of 1, 5, and 10% MeOH in $CHCl_3$, 50 mL of 15% MeOH in $CHCl_3$ (collected in four separate fractions), and 25 mL of 20% MeOH in $CHCl_3$. The desired product, **6**, eluted in fractions 3 and 4 of the 15% MeOH cut. Concentration of these combined fractions gave pure **6** as a colorless semisolid (yield 3.9 mg, 33%). The integrity of the aldehyde group of reacylated **6** was established by comparison of the C=O stretch region in the IR spectrum of reacylated **6** with that in the IR spectra of authentic **6**, **3**, and phosphatidylglycerol (PG, Sigma): **6**, 1690 (aldehyde) and 1720 cm^{-1} (ester); **3**, 1680 cm^{-1}; PG, 1735 cm^{-1}; reacylated **6**, 1695 (aldehyde) and 1735 cm^{-1} (ester). Fraction 2 of the 15% MeOH cut contained material (2.7 mg) with an R_f value only slightly higher than that of **6**. However, the characteristic aldehyde C=O stretch was completely absent in this material, indicating that some breakdown of the benzaldehyde head group had occurred during reacylation.

To minimize this unwanted side reaction, reacylation of the monoacyl compound with 14-proxyl[^{3}H]stearic acid was carried out at a lower temperature as follows: 14-proxyl[2-^{3}H]stearic acid (9.1 mg, dried under high vacuum; Keana & Boyd, 1981) and CDI (4.0 mg) were dissolved in 0.1 mL of dry $CHCl_3$ and kept at room temperature under dry nitrogen for 30 min. The mixture was added to 8.2 mg of the monoacylbenzaldehyde, and the resulting solution was kept at 40 °C under a stream of dry nitrogen. TLC indicated that the reaction was essentially complete after 3 h. Workup and purification similar to that described for reacylated **6** yielded pure [^{3}H]-**7** (5.2 mg, 39%, specific radioactivity 1.716×10^7 cpm/μmol) as a pale yellow waxy semisolid. The breakdown product observed during the pilot reacylation was not apparent in this preparation.

Sodium Methyl 4-[^{3}H]Formylphenyl Phosphate ([^{3}*H*]-**5**). To a stirred solution of dichloridate **8** (429 mg) in dry ether (6 mL) containing dry pyridine (1.5 mL) at 0 °C was added slowly dry MeOH (1.25 mL). After a 1-h stir, the reaction mixture was allowed to warm up and refluxed for 1 h. Concentration gave a pale yellow oil that was redissolved in ether, washed with 1 N H_2SO_4 and water, and then dried ($MgSO_4$). The combined aqueous layers were saturated with NH_4Cl, acidified with H_2SO_4, and back-extracted with ether. Concentration of the combined ether layers gave crude dimethyl 4-formylphenyl phosphate, which was purified by preparative TLC with 4% MeOH in $CHCl_3$ as the solvent (yield 203 mg, 57%): NMR ($CDCl_3$) δ 3.80 (d, 6 H), 7.38 (d, 2 H), 7.88 (d, 2 H), and 9.97 (s, 1 H).

A solution of dimethyl 4-formylphenyl phosphate (29 mg) in dry 2-propanol (1.5 mL) was added to 2.7 mg of solid sodium boro[^{3}H]hydride (116.6 Ci/mol, New England Nuclear). After 3 days at 25 °C under nitrogen, ethylene glycol (0.076 mL) was added, and after 30 min, the solution was concentrated to dryness, and the residue was extracted with ether. Concentration gave pure dimethyl 4-(hydroxy[^{3}H]-methyl)phenyl phosphate (20.7 mg, 71%) as a colorless oil. A cold sample was similarly prepared. NMR ($CDCl_3$) showed the following: δ 3.80 (d, 6 H), 4.60 (s, 2 H), and 7.20 (m, 4 H).

The tritium-labeled alcohol (20.7 mg) dissolved in dry CH_2Cl_2 (0.9 mL) was added to pyridinium chlorochromate (41 mg) in dry CH_2Cl_2 (0.4 mL) and stirred for 4 h at room temperature. Ether (20 mL) was added, and the solution was filtered through a plug of anhydrous $MgSO_4$. Concentration of the filtrate gave dimethyl 4-[^{3}H]formylphenyl phosphate (16.7 mg, 81%) as a yellow oil.

To a solution of dimethyl 4-[^{3}H]formylphenyl phosphate (16.7 mg) in dry methyl ethyl ketone (1 mL) was added NaI (14.1 mg), and the resulting solution was refluxed for 40 min. The solution was then cooled to 0 °C, and a white precipitate was removed by centrifugation and washed with cold solvent. The combined mother liquor and washes were concentrated to two-thirds of the original volume and placed in the freezer for a second crop. The combined crops were purified by preparative TLC with $CHCl_3$/MeOH/water (4:1.5:0.1) as the solvent, giving pure [^{3}H]-**5** (yield 14.9 mg, 86%; specific activity 2.124×10^7 cpm/μmol). A cold sample was similarly prepared in a 85% yield. Anal. Calcd for $C_8H_8O_5PNa \cdot {}^1/_4H_2O$: C, 39.73; H, 3.69. Found: C, 39.61; H, 3.53. NMR (CD_3OD) showed the following: δ 3.70 (d, 3 H), 7.44 (d, 2 H), 7.88 (d, 2 H), and 9.90 (s, 1 H).

Materials. Lipid-depleted beef heart cytochrome *c* oxidase in 1% cholate, 0.25 M sucrose, 50 mM phosphate, and 1 mM EDTA (SPE), pH 7.4, was prepared by the method of Yu et al. (1975) and kindly supplied by Dr. Tsoo E. King. The heme *a* and phospholipid contents of the preparation were determined to be 10.3 nmol of heme *a*/mg of protein and 60 nmol of lipid phosphorus/mg of protein, respectively. Cytochrome *c* oxidase, with an activity of 15 nmol of O_2 s^{-1} (nmol of heme *a*)$^{-1}$ [by the method of Yonetani (1966)], was stored at 17.7 mg of protein/mL in 1% cholate/SPE, pH 7.4, at −196 °C. Before use, the protein was dialyzed overnight against 1% cholate/SPE, pH 6.5, to remove any residual ammonium sulfate. L-α-Dioleoylphosphatidylcholine (99% pure) was from Sigma. Cholic acid (Sigma) was recrystallized twice from ethanol. All other chemicals were obtained from commercial sources and were of reagent grade or better.

Assays. Protein was determined by the method of Lowry (1951) as modified by Miller (1959) using bovine serum albumin (Pentex fatty acid poor fraction V) with $E^{1\%}_{279} = 6.67$ (Sterman & Foster, 1956) as the standard. Heme *a* was determined by the method of Yonetani (1961) and Griffiths & Wharton (1961). The phospholipid content of lipid/protein samples was measured by lipid phosphorus determination after extraction of the lipids by the method of Bligh & Dyer (1959) in the presence of ammonium hydroxide (Awasthi, 1971). Lipid phosphorus determination was essentially as described by Lowry & Tinsley (1974) except that lipids were digested for 6 h at 140 °C.

Covalent Labeling of Cytochrome c Oxidase. The method of addition of the aldehyde label depended on its aqueous solubility. For the phospholipid aldehydes, **6** and **7**, stock solutions of the label and of dioleoylphosphatidylcholine (DOPC) in chloroform were mixed in a small glass, Potter-type homogenizer to give the desired molar ratio. The solvent was removed under a stream of nitrogen followed by evacuation for 1 h. The dry lipids were then taken up into 1% cholate/SPE, pH 6.5, containing 10 mg of cytochrome *c* oxidase/mL, and after gentle hand homogenization, carefully avoiding the formation of bubbles, the solution was allowed to equilibrate at room temperature for 1 h. The sample was transferred to a Schleicher & Schuell M_r 75 000 cut-off collodion bag, and the cholate was removed by dialysis against SPE, pH 6.5, buffer at 4 °C (three changes of buffer followed by dialysis overnight), resulting in cytochrome *c* oxidase reconstituted into phospholipid bilayers. This method gives lamellar vesicles as judged by electron microscopy of negatively stained preparations. After homogenization, a small aliquot was withdrawn for determination of the molar ratios of cytochrome *c* oxidase, DOPC, and aldehyde. Buffer was added to the remaining sample to give a final protein concentration of 6 mg/mL. The sample was divided into two, and sodium cyanoborohydride was added to one aliquot to a final concentration of 100 mM, while a corresponding volume of buffer was added to the second aliquot. The latter served as a control to determine that there was no irreversible covalent attachment of the label to the protein in the absence of the reducing agent. Both reaction mixtures were stirred at 4 °C for 18–20 h. The reactions were quenched by the addition of hydroxylysine to a final concentration of 10 mM, followed by stirring in the cold for 1 h. The procedure followed for covalent labeling of cytochrome *c* oxidase with phospholipid **1** was similar in overall design to that described here for **6** and **7** but differed in some details for historical reasons. For example, the cosolubilized mixture of cholate, **1**, DOPC, and cytochrome *c* oxidase was dialyzed against SPE, pH 6.0, to form the vesicular lipid/protein sample, and after the addition of sodium cyanoborohydride, the reaction was allowed to incubate at 37 °C for 1 h, instead of 4 °C overnight, and no additional primary amine was added.

The single-tail acetaldehyde label **2** was reconstituted with cytochrome *c* oxidase and DOPC as described for **6** and **7**. Reaction of the sample with and without 100 mM sodium cyanoborohydride was achieved with stirring at either 4 °C overnight or 37 °C for 1 h. The reaction was quenched by the addition of 10 mM lysine and 50 mM ammonium sulfate in the dissociation buffer used to prepare the samples for SDS–polyacrylamide gel electrophoresis (see below).

For the single-tail benzaldehyde labels **3** and **4**, cytochrome *c* oxidase was first reconstituted into DOPC bilayers, and the vesicle suspension was homogenized and then added to a dry film of **3** or **4**. The sample was rehomogenized and equilibrated for 1 h at room temperature, and the reaction was allowed to proceed in the presence and absence of sodium cyanoborohydride as described for **6** and **7**. For the water-soluble benzaldehyde **5**, the reaction mixture was similarly prepared, except that addition of the cytochrome *c* oxidase/DOPC vesicle suspension to dry **5** was followed by bath sonication for 1 h at 0 °C prior to the addition of the reducing agent.

Removal of Excess Aldehyde Label. To obtain the ESR line shape of the lipid spin-label **7** covalently attached to cytochrome *c* oxidase in DOPC, it was necessary to remove as much of the residual noncovalently attached spin-label as possible. For this purpose, the reaction mixture was brought to 2% cholate by addition of 20% cholate in distilled water, homogenized, and allowed to equilibrate for 1 h at room temperature. This solution was then layered on top of 3–4

mL of 2% cholate/SPE buffer (pH 7.4) containing 20% sucrose, which was layered over a pad (0.5 mL) of the same buffer containing 60% sucrose. After a 18–20-h spin at 35000 rpm and 4 °C a SW 50.1 rotor, the protein band that had formed on top of the 60% sucrose pad was collected, and excess sucrose was removed by dialysis against 1% cholate/SPE, pH 7.4. The cholate concentration was then restored to 2%, DOPC was added back to give a lipid to protein molar ratio of about 100, and the sample was recentrifuged as above and dialyzed against 1% cholate/SPE, pH 7.4. This procedure was repeated twice more except that no DOPC was added prior to the final centrifugation step. Samples for ESR were then prepared by addition of the appropriate amount of DOPC followed by dialysis to remove the cholate. The efficiency of the delipidation procedure (i.e., removal of the original lipids present during the covalent labeling) was tested by adding ^{14}C-labeled phosphatidylcholine (0.01 mCi, 0.14 mg, New England Nuclear) to the sample prior to the centrifugations and by monitoring the removal of ^{14}C counts in the cytochrome *c* oxidase containing fractions. SDS–PAGE of the delipidated protein showed, however, that some noncovalently attached **7** was retained, amounting to approximately 40% of the total spin-label remaining, while the [^{14}C]PC was reduced to background level after only two centrifugation steps. ESR spectra were recorded at this stage and again after the amount of unattached spin-label was further reduced to about 15% with a series of ammonium sulfate precipitations in the presence of cholate by the method of Yu et al. (1975). The difficulty in removing the excess **7** and its derivative may be related to the fact that negatively charged lipids exhibit a stronger association with cytochrome *c* oxidase than does the zwitterionic PC (Marsh & Watts, 1982).

Preparation of Samples for ESR Reference Spectra. To obtain the bilayer reference line shape of **7** and to compare the behavior of **7** covalently attached to cytochrome *c* oxidase and **7** free to equilibrate between the lipid/protein interface and the bilayer, the reactive aldehyde of **7** was blocked by reaction with hydroxylysine. The aldehyde **7** (0.15 μmol) and DOPC (14.85 μmol) dissolved in chloroform were mixed, the solvent was removed under vacuum, 0.181 mL of buffer (SPE, pH 6.5) containing 100 mM hydroxylysine and 1 M sodium cyanoborohydride was added, and the mixture was incubated with stirring at 4 °C for 18 h. Bilayer reference ESR spectra were obtained by diluting an aliquot (15 μL) of the lipid mixture with 45 μL of SPE, pH 7.4. Other aliquots of the vesicles containing the hydroxylysine-conjugated **7** and DOPC were used for reconstitution with cytochrome *c* oxidase as follows. The lipids were solubilized in 1% cholate/SPE, pH 7.4, and cytochrome *c* oxidase (10 mg/mL) in 1% cholate/SPE, pH 7.4, was added to give the desired lipid/protein ratio. The sample was homogenized and allowed to equilibrate for 1 h at room temperature and cholate removed by dialysis against SPE, pH 7.4, as described earlier.

Electron Spin Resonance Spectroscopy. ESR spectra were recorded on a Varian E-9 9.5-GHz spectrometer interfaced with a 32K Varian 620/L100 computer for spectral analysis. ESR instrument settings were as follows: microwave power 5 mW, scan time 16 min, scan range 100 G, filter time constant ≤0.2 s, and modulation amplitude ≤1 G. Sample temperature was maintained within ±0.2 °C and monitored with a Fluke digital thermometer and copper–constantan thermocouple located 1 cm above the sample in the ESR cavity. Spectral subtractions and data analysis were performed as described previously (Jost & Griffith, 1978; Brotherus et al., 1980; Silvius et al., 1984).

SDS–Polyacrylamide Gel Electrophoresis. Disc gel electrophoresis was performed essentially by the method of Swank & Munkres (1971). Gels contained 10% acrylamide, 0.66% bis(acrylamide), 8 M urea, 0.1% SDS, and 100 mM phosphoric acid, adjusted to pH 6.8 with Tris base. Cytochrome *c* oxidase samples (5 mg of protein/mL) were dissociated by addition of an equal volume of a buffer containing 10% SDS, 8 M urea, 40 mM sodium phosphate, and 10% β-mercaptoethanol, pH 6.8, followed by heating at 37 °C for 1 h. Marker dye (methyl green) and sucrose were added to the dissociated protein samples to final concentrations of 0.05 and 3%, respectively. To each gel 100 μg of protein was applied, and electrophoresis was performed at 2 mA/gel for 1 h followed by 6–7 h at 4 mA/gel. Gels were stained with 0.25% Coomassie Blue R-250 (Eastman Kodak) in methanol/water/glacial acetic acid (5:4:1 by volume) at 60 °C for 30 min. Destaining was achieved in methanol/water/glacial acetic acid (5:4:1 by volume) with Bio-Rad analytical-grade mixed-bed resin AG 501-X8 (20–50 mesh) at room temperature with gentle shaking. Gels were swollen in 10% acetic acid and scanned at 550 nm in a Beckman DU spectrometer equipped with a Gilford linear transport attachment. Gels were then cut into 1-mm slices (Hoeffer gel slicer), and each slice was placed in a scintillation vial. The slices were dissolved in 1 mL of 15% H_2O_2 at 70 °C overnight. Aquassure (7 mL, New England Nuclear) was added, and radioactivity was measured in a Beckman LS7000 liquid scintillation counter.

RESULTS

Syntheses of Aldehyde Labels. The generation of aldehyde **1** was approached by periodate oxidation of the corresponding phosphatidylglycerol, while aldehyde **2** was prepared by a multistep procedure starting with bromoacetaldehyde diethyl acetal. Of greater utility in this study, however, were the reactive benzaldehyde-containing molecules **3**–**7**. Compounds **3**–**6** were obtained via coupling of the corresponding alcohols with phosphorodichloridate (**8**) as the key step, as indicated in Figure 2. In general, the synthetic procedures were first worked out, and satisfactory analysis was obtained with nonradiolabeled starting materials. The radiolabeled forms of **5** and **6** were obtained through reduction of cold **5** and **6** with tritiated sodium borohydride followed by oxidation with pyridinium chlorochromate. The advantage of this procedure is that the radiolabel is introduced in the final steps of the syntheses. The resulting tritium on the aldehyde carbon atom, or the corresponding reduced benzylamine derivative, does not exchange under the conditions employed in these experiments. [^{3}H]-**7** was prepared from cold **6** via hydrolysis with phospholipase A_2 followed by reacylation with tritiated 14-proxylstearic acid. The final labeling reagents **3**–**7** were obtained in satisfactory yields and were found to be very stable by the criterion that no significant decrease in labeling efficiency was observed for stock solutions in organic solvents over a period of several months at −20 °C.

Labeling of Cytochrome c Oxidase. Our first attempt to achieve amine-specific labeling of cytochrome *c* oxidase involved the spin-labeled phosphatidylglycolaldehyde **1** reconstituted with cytochrome *c* oxidase and DOPC at a molar ratio of protein:DOPC:**1** of 1:50:35 and reacted in the presence of sodium cyanoborohydride. ESR line shapes obtained of the sample after three delipidations by centrifugation through 2% cholate/20% sucrose in SPE, pH 7.4, demonstrated attachment of the label to the protein. However, the ESR spectra indicated that the covalently attached label was slowly released with time even after reduction with sodium cyanoborohydride. In addition, very substantial retention of the label after delipidation

was also observed when the reduction with sodium cyanoborohydride was omitted. SDS–polyacrylamide gel electrophoresis of cytochrome *c* oxidase samples reacted with the radiolabeled phosphonate aldehyde **2** (130 nmol of DOPC:45 nmol of **2**:1 nmol of protein) showed that this molecule also bound to the protein in the absence of the reducing agent although the level of attachment appeared higher when the sodium cyanoborohydride was included. The gel pattern obtained for cytochrome *c* oxidase reacted with aldehyde **2** both with and without sodium cyanoborohydride showed significant labeling of bands V and VII and minor labeling of bands III, IV, and VI.

The unexpected behavior of aldehydes **1** and **2**, i.e., irreversible attachment to the protein even in the absence of sodium cyanoborohydride, raises some questions concerning the chemistry of these aldehydes. NMR results obtained for **1** showed spectral properties that were not consistent with the presence of a free aldehyde group, although the NMR spectrum of phosphonate aldehyde **2** did indicate the presence of a free aldehyde group. Perttilä (1984) reported the generation of an unlabeled phosphatidyl glycoaldehyde by periodate oxidation but did not report characterization of the compound. In an attempt to clarify this point, we prepared hexadecylglycero-1-phosphate and subsequently oxidized it with sodium periodate by the procedure used to generate **1**. The product did not show an aldehyde proton peak in the NMR spectrum and did not readily undergo reductive amination with benzylamine and sodium cyanoborohydride in aqueous solution at pH 6. For these reasons, **1** and **2** were judged to be unsatisfactory for the purposes of this study. The benzaldehyde labels **3**–**7**, however, demonstrated the proper chemical behavior; i.e., the NMR spectra exhibited a free aldehyde group, and irreversible covalent attachment to the protein occurred only in the presence of sodium cyanoborohydride.

ESR Spectra and Molecular Dynamics of the Lipid Labels. Of the five benzaldehyde molecules prepared, two (**4** and **7**) were spin-labeled. The single hydrocarbon tail molecule **4** was synthesized to examine the effect of introduction of the nitroxide moiety in the hydrocarbon tail on the label distribution pattern, rather than to obtain information about molecular dynamics. The nitroxide group is attached very close to the hydrocarbon end of the acyl chain of **4**, which results in a very narrow ESR line shape that is of limited usefulness in distinguishing different motional environments. In molecule **7** the nitroxide moiety is at the 14-position of the 18-carbon tail, which is known from previous studies to be sufficiently removed from the terminus to provide a sensitive indicator of the local envirnoment of the unpaired spin (Jost & Griffith, 1980). A series of ESR spectra was obtained at 25 °C for the spin-labeled phospholipid analogue **7** covalently attached to cytochrome *c* oxidase in bilayers with DOPC at lipid:protein ratios ranging from 35 to 280. A comparable series of spectra was recorded for the hydroxylysine derivative of **7** free to equilibrate between the bulk lipid and the surface of cytochrome *c* oxidase. Reference spectra of **7** in DOPC bilayers were obtained at a series of temperatures (−4 to 25 °C) to determine the motional characteristics of **7** in the absence of protein.

Representative ESR spectra are shown in Figure 3. A typical spectrum of **7** after reaction with cytochrome *c* oxidase and removal of most of the free aldehyde is shown in Figure 3A. Reference bilayer line shapes at two temperatures are superimposed in Figure 3B. Figure 3C is the motion-restricted component representing label in contact with the surface of the protein. When the motion-restricted component is subtracted from the composite spectrum of Figure 3A, the difference line shape matches a bilayer line shape obtained at 10 °C (dashed line of Figure 3B). The splitting measured from the outermost peaks ($2A_{max}$) of Figure 3C is 60 G. This is the same, within experimental error, as the corresponding splitting measured for the motion-restricted component of the hydroxylysine derivative of **7** when it is free to equilibrate between the lipid and the surface of the protein, but is significantly less than the splitting observed for aggregated, delipidated cytochrome *c* oxidase (65 G at 12 mol of phospholipid/mol of protein).

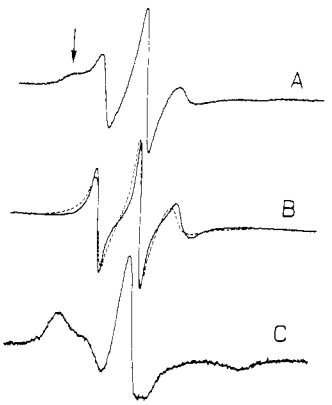

FIGURE 3: ESR spectra of the phospholipid benzaldehyde spin-label **7** at 25 °C (with samples in SPE buffer, pH 7.4). (A) **7** covalently attached to cytochrome *c* oxidase (1.4 mol/mol of protein) and reconstituted with DOPC at a DOPC:protein ratio of 113:1. Free, unattached, **7**, has been removed by a series of four centrifugations through 2% cholate/20% sucrose, followed by four ammonium sulfate precipitations. As judged by SDS–PAGE, about 85% of the spin-label is attached to the protein. (B) The hydroxylysine derivative of **7** (1 mol %) free to diffuse in DOPC bilayers at 25 (solid line) and 10 °C (dashed line). (C) The line shape of the motion-restricted component of (A) (see arrow). This line shape, which was used in all spectral subtractions, was obtained by incremental subtraction of reference bilayer line shapes [see Silvius et al. (1984)] obtained at a series of temperatures from a composite spectrum similar to (A) but with a lower lipid to protein ratio (34:1). The reference bilayer line shape that gave the best match to the fluid component of the composite spectrum, and was used to obtain (C), was for the hydroxylysine derivative of **7** in DOPC at −4 °C.

The fraction of spin-label in contact with the protein, F_c, operationally defined as the double integral of the motion-restricted ESR spectral component divided by the double integral of the entire spectrum, is plotted in Figure 4 as a function of the lipid:protein molar ratio. The open circles are data points for the hydroxylysine derivative of **7**. This molecule, which has a blocked reactive group, does not covalently attach to the cytochrome *c* oxidase, as was confirmed by SDS–PAGE. As expected, F_c decreases rapidly with increasing lipid content of the samples (open circles, Figure 4). In order to determine F_c for the samples containing **7** covalently attached to cytochrome *c* oxidase, spectral subtraction was used in combination with information from the gel data to correct F_c for the contribution of noncovalently bound labels still present after delipidation. The results are plotted in Figure 4 (shaded symbols). In this case the fraction of motion-restricted ESR spectral component is approximately independent, within experimental error, of the lipid to protein ratio.

Distribution of the Benzaldehyde Labels Covalently Linked to Cytochrome c Oxidase. After reaction of the aldehyde labels with cytochrome *c* oxidase reconstituted in phospholipid vesicles and quenching unreacted excess reagent with hydroxylysine, SDS–polyacrylamide gels were run to examine the distribution of labels covalently attached to the cytochrome *c* oxidase polypeptides. The labeling patterns observed for the

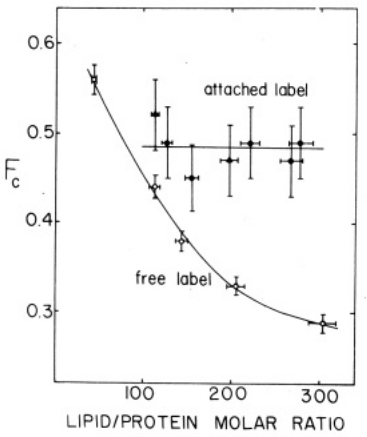

FIGURE 4: Fraction of the motion-restricted component (F_c) of **7** in cytochrome *c* oxidase preparations as a function of the lipid content. The open circles are data for the spin-label free to diffuse in the bilayer (the hydroxylysine derivative of **7**). The filled symbols (●, ▲) are derived from ESR spectra of **7** covalently linked to cytochrome *c* oxidase, after correction for the presence of unattached **7**. There are two sets of data. The filled circles are for a preparation that retained 40% of unattached residual spin-label, and the filled triangles are for the same preparation after the residual unattached **7** was reduced to 15%. The correction was made with the relationship $F_c^{total} = AF_c^{att} + (1 - A)F_c^{unatt}$, where A is the fraction of covalently attached **7** present in the sample, which was estimated from the gel patterns by integrating the radioactivity in the protein bands and in the free lipid peak. F_c^{total} is the fraction of the motion-restricted component in the composite spectrum. F_c^{att} is the fraction of the motion-restricted component for covalently bound **7** (filled symbols). F_c^{unatt} is the fraction of the motion-restricted component for unbound **7** and is estimated from the independent set of data for the hydroxylysine conjugate of **7**. The vertical error bars for the data of covalently attached **7** are estimates arrived at by computing the square root of the sum of the squares of the independent errors of each of the quantities used in the correction.

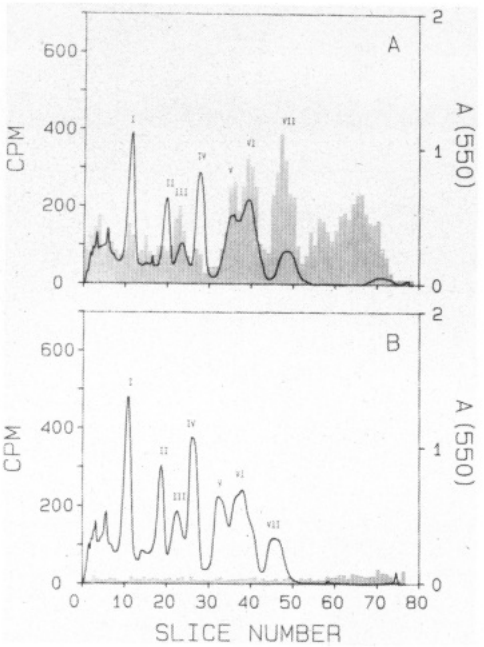

FIGURE 5: (A) Labeling of cytochrome *c* oxidase with the radioactive single lipid chain benzaldehyde label **3**. (B) Control where the reducing agent, sodium cyanoborohydride, was omitted. In each plot the line is the optical densitometer tracing of Coomassie Blue stained SDS–polyacrylamide gels of cytochrome *c* oxidase, and the superimposed bar graph represents the distribution of the radioactive labels. The direction of migration is to the right. The molar ratios of label: phospholipid:protein in the reaction mixtures were 18:93:1 for (A) and (B).

single hydrocarbon tail benzaldehyde molecule **3** in the presence and absence of the reducing agent shows that per-

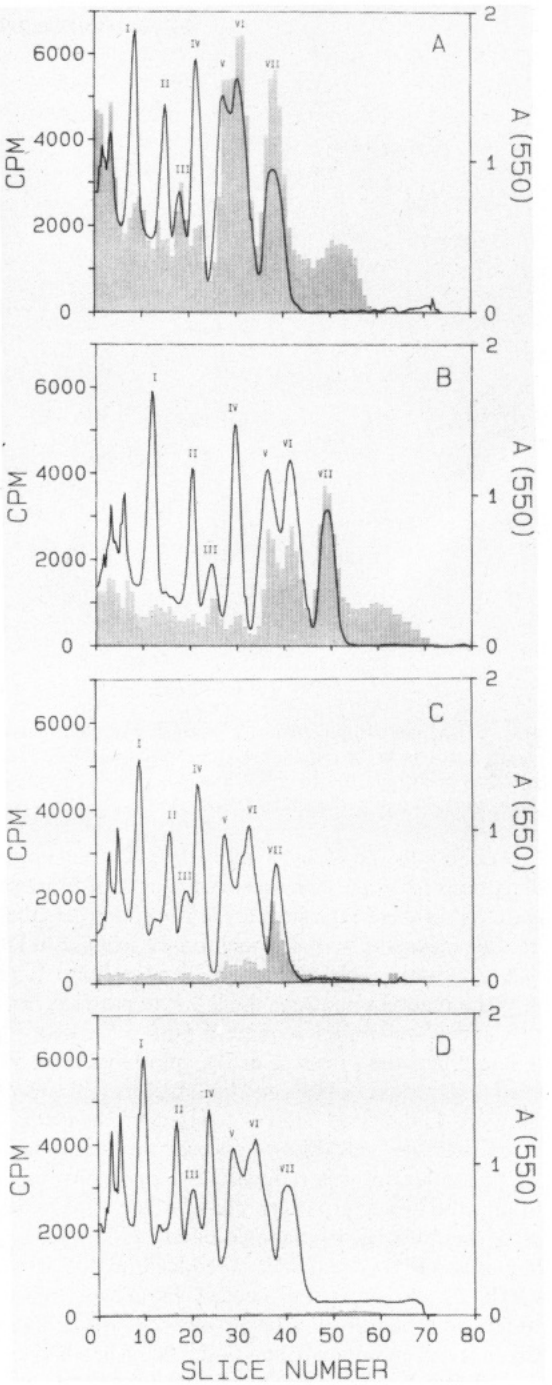

FIGURE 6: (A–C) Labeling of cytochrome *c* oxidase with **4**, the spin-labeled analogue of **3** as a function of the benzaldehyde to protein molar ratio. The line and bar graphs are as in Figure 5. The molar ratios of label:phospholipid:protein in the reaction mixtures were (A) 23:130:1, (B) 15:290:1, and (C) 3.4:300:1. (D) Control, run under the same conditions as (A), except that the sodium cyanoborohydride was omitted.

manent covalent attachment requires sodium cyanoborohydride in the reaction (Figure 5). This experiment was done in two ways. In one experiment the reconstitution of cytochrome *c* oxidase in DOPC vesicles was carried out in the presence of the aldehyde label **3** (results shown in Figure 5). In the second experiment, the protein was first reconstituted in DOPC vesicles, and the aldehyde label was added subsequently. The results were similar to those shown in Figure 5. The most heavily labeled protein band is VII while bands III, V, and VI are also significantly labeled. Only minor quantities of radioactivity appear to be associated with bands I, II, and IV. The broad peak to the right of band VII is residual unattached

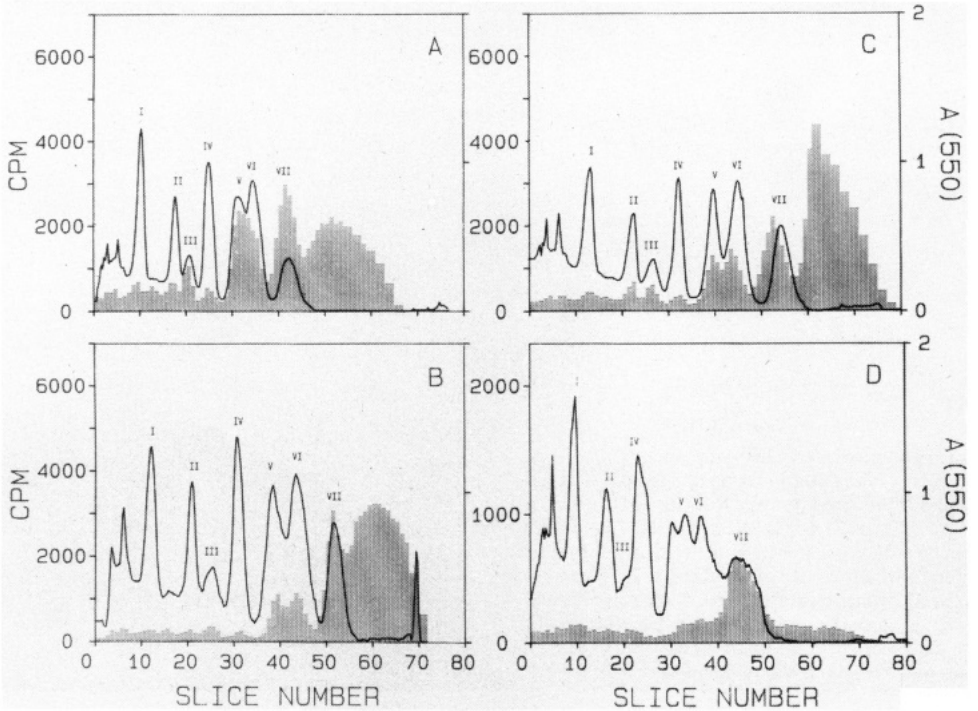

FIGURE 7: Labeling of cytochrome *c* oxidase with the phospholipid labels **6** (A and B) and **7** (C and D). The molar ratios of the label: phospholipid:protein in the reaction mixture were (A) 28:82:1, (B) 19:318:1, (C) 21:86:1, and (D) 4.8:334:1. Panel D differs in that the unreacted aldehyde has been removed for ESR analysis (see Figure 3A). The lines and superimposed bar graphs are as in Figure 5, but note that the cpm scale for (D) is not the same as for (A–C).

label, probably in the form of its hydroxylysine conjugate.

Gel patterns for **4**, the spin-labeled analogue of **3**, are shown in Figure 6. In these experiments the benzaldehyde label was added after reconstitution of cytochrome *c* oxidase in DOPC vesicles. At comparable benzaldehyde:phospholipid:protein ratios in the reaction mixture, the labeling patterns for molecules **3** and **4** are similar (compare Figure 5A with Figure 6A). Therefore, the presence of the spin-label moiety does not have a significant effect on the label distribution.

Figure 6A–C shows an interesting effect on the gel patterns that occurs as the benzaldehyde:protein ratio is decreased. When the amount of **4** is reduced, less incorporation is observed and the labeling pattern changes. In Figure 6B, for example, the most heavily labeled band is still VII, but the labeling of bands III, V, and VI is reduced relative to that of band VII. This trend is continued in Figure 6C, where the benzaldehyde:protein ratio is even lower. Only at high benzaldehyde levels is some labeling observed of bands I, II, and IV, but the radioactivity associated with these bands drops to background levels as the benzaldehyde level is reduced (Figure 6C). The change in the appearance of the labeling patterns with varying benzaldehyde:protein ratios points out the importance of examining a series of labeling ratios, instead of reporting the results of only one reaction condition. The phospholipid content of the samples was increased in proceeding from panel A to panels B and C of Figure 6, with no significant effect on the trend in labeling patterns as the amount of benzaldehyde label was decreased.

The control data of Figure 6D show that no labeling occurs when the reducing agent, sodium cyanoborohydride, is omitted. This absolute dependence of permanent covalent labeling on the presence of the reducing agent was observed for all of the benzaldehyde molecules used in this study.

Labeling patterns for the benzaldehyde phospholipid analogues **6** and **7** are shown in Figure 7. The spin-labeled and non-spin-labeled phospholipid analogues gave similar results. In these experiments, the benzaldehyde labels were introduced

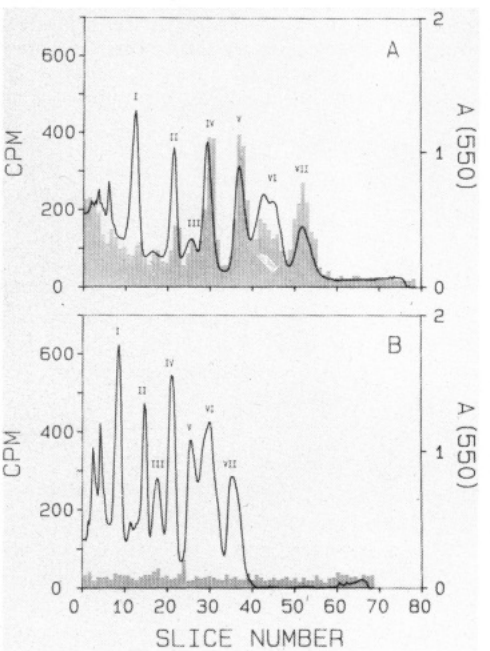

FIGURE 8: (A) Labeling of cytochrome *c* oxidase with the water-soluble benzaldehyde label, **5**. (B) Control omitting the sodium cyanoborohydride reduction step. The lines and superimposed bar graphs are as in Figure 5. The molar ratio of the water-soluble label: phospholipid:protein during the reaction was (A and B) 300:93:1.

along with the DOPC in the reconstitution step. The heaviest labeling occurs in band VII. There is substantial labeling of bands V and VI and some labeling of band III at high benzaldehyde:protein ratios. When the benzaldehyde:protein ratio is lowered, there is a reduction in the labeling of bands III, V, and VI relative to band VII, as was observed with the single-tail label **4**. The free lipid peak in Figure 7D is largely removed as a result of repeated centrifugation and ammonium sulfate precipitation. This is the same sample used to obtain the ESR spectrum shown in Figure 3A. The controls for both

6 and 7 showed that no significant radioactivity was retained on cytochrome *c* oxidase when the reducing agent was absent.

The labeling pattern observed for the water-soluble benzaldehyde 5 (Figure 8) differs significantly from those of the lipid derivatives 3, 4, 6, and 7. In this case, band IV along with bands V and VII is heavily labeled, and bands II, III, and VI exhibit some labeling. The control (Figure 8B) shows that for 5, as well as for the lipid derivatives, the reduction step is necessary to obtain permanent covalent attachment to the protein.

Integrating the labeling patterns provides information regarding the extent of reaction of the benzaldehyde labels with cytochrome *c* oxidase. From the data of Figure 7A, for example, integration over bands I–VII yields a total radioactivity of 43 000 cpm, while the free lipid peak to the right contains 33 000 cpm. The number of counts loaded on the gel was on the order of 235 000 cpm so that the recovery of total counts (all peaks combined) was 32%. The recovery for the lipid labels ranged from 20 to 45%, with the recovery for the phospholipid derivatives 6 and 7 on the upper end of this range. The recovery for the water-soluble benzaldehyde 5 was much lower (about 2%). The recovery is inversely related to the aqueous solubility of the benzaldehyde molecules because part of the labels present in the free lipid peak are lost during staining and destaining of the gels.

Of more interest is an estimate of the labeling efficiency. The amount of cytochrome *c* oxidase loaded on the gel was roughly 100 μg, and the specific activity of label 6 was 16 800 cpm/nmol. Combining these numbers, we estimate 5.2 mol of label covalently attached per mole of cytochrome *c* oxidase. Thus, for the sample of Figure 7A, which had a molar ratio of label:phospholipid:protein in the reaction mixture of 28:82:1, the labeling efficiency was about $5.2/28 \times 10^2 = 20\%$. This value is typical for the lipid labels 3, 4, 6, and 7 (data of Figures 5–7). The number of moles of label incorporated per mole of protein ranged from 1 to 6, depending on the molar ratios of benzaldehyde:lipid:protein. For the water-soluble label 5, the labeling efficiency was about 2%, which is not surprising considering that this label is distributed throughout the aqueous phase, rather than being concentrated in the membraneous sample. However, the number of labels incorporated per mole of protein was 6, as estimated from the data of Figure 8A.

Discussion

In this study we sought to prepare and use a series of labels that would be expected to show a high degree of specificity toward amino groups accessible to phospholipid head groups. An aldehyde was chosen as the reactive functional group, reasoning that Schiff base formation would be selective toward lysine residues (and N-terminal α-amino groups) and be reversible until addition of the reducing agent sodium cyanoborohydride as diagrammed:

$$\text{protein—NH}_2 + \text{O=CH—R} \rightleftharpoons$$
$$\text{protein—N=CH—R} \xrightarrow{\text{NaBH}_3\text{CN}} \text{protein—NH—CH}_2\text{—R}$$

Our first approach involved the synthesis of spin-labeled phosphatidylglycerol and subsequent periodate oxidation to give phosphatidyl glycolaldehyde (1). In our hands, 1 did not exhibit the NMR spectral properties of an aldehyde, possibly because it existed largely in a cyclic hemiacetal form. Furthermore, although 1 did couple to cytochrome *c* oxidase, the reaction was not readily reversible when the cyanoborohydride reduction was omitted. With the aim of preparing a label with a well-defined aldehyde group, we turned next to the synthesis of the phosphonate aldehyde 2. Because cyclization of the P–OH group into the aldehyde group of this molecule would involve formation of a strained four-membered ring, it seemed likely that the free aldehyde would be the favored form. However, 2 also irreversibly bound to cytochrome *c* oxidase whether or not sodium cyanoborohydride was added. A new aldehyde was therefore required, preferably in the phosphate series. We chose to prepare a series of benzaldehyde labels with the phosphate substituted at the 4-position. It is geometrically impossible for the aldehyde group to cyclize with the P–OH group attached to the 4-position in these compounds. Five molecules were synthesized: 3 and 4 are lipid-like molecules with single hydrocarbon tails, 6 and 7 are phospholipid analogues, and 5 is a water-soluble benzaldehyde used as a control (see Figure 1). All five of these molecules satisfied the criterion of forming stable covalent bonds with the protein only after reduction with sodium cyanoborohydride.

Several interesting observations can be made from the chemical labeling patterns obtained for cytochrome *c* oxidase. First, the presence of the spin-label does not affect the labeling pattern observed with either the single hydrocarbon tail or phospholipid molecules. Apparently, the nitroxide does not significantly alter the position of the reactive aldehyde groups with respect to the protein surface. Second, essentially the same labeling patterns are observed with both the single-tail and phospholipid aldehydes. With the single-tail lipids, especially at the higher labeling ratios, there is the possibility of labeling of parts of the protein exposed to the aqueous environment because these labels may not be totally incorporated into the bilayer. However, by inclusion of the phospholipid labels 6 and 7, which have negligible water solubility, this potential artifact has been ruled out.

The presence of the nitroxide group makes possible spectroscopic measurements to obtain additional information regarding the location and environment of the covalently attached phospholipid analogues. ESR of the spin-labels is a sensitive indicator of molecular dynamics. The ESR line shapes of the nitroxide moiety have been extensively studied, and it is easy to distinguish the sharp three-line spectrum characteristic of the spin-label rapidly tumbling in aqueous solution from the same spin-label in a phospholipid bilayer or in contact with the hydrophobic surface of a membrane protein (Marsh & Watts, 1982; Jost & Griffith, 1980). It has been suggested that some membrane protein labeling reagents based on aryl azides, for example, can loop back and label the protein from the aqueous phase rather than from the bilayer (Bayley & Knowles, 1978). The ESR spectra recorded before and after reaction rule out this possibility in the case of the benzaldehyde labels because no aqueous component is detected. Furthermore, the ESR spectra of the spin-labeled phospholipid 7 covalently attached to cytochrome *c* oxidase show that about 50% of the nitroxide-carrying acyl chains of 7 are in intimate contact with the protein and this fraction is relatively constant as the lipid content of the sample is increased (Figure 4). The line shape and splitting of this motion-restricted component are similar to that of unattached lipid spin-labels contacting the protein in a dynamic equilibrium between protein surface and bulk bilayer, indicating that the restriction of acyl chain motion is similar for the spin-label covalently attached to the protein surface or in transient contact with the protein. The second, more mobile component observed in the ESR spectra of 7 covalently attached to cytochrome *c* oxidase resembles that of lipid spin-labels in pure lipid bilayers or the bilayer component of lipids free to diffuse in protein-containing bilayers, except that the lines are broader due to the influence

of the nearby cytochrome *c* oxidase. Because the covalently attached label is unable to diffuse away from the protein surface, it experiences an environment that is on the average more motion restricted than is the case with a freely diffusable spin-label, which can sample positions in the bilayer further removed from the protein surface.

The gel electrophoresis data show that the labeling pattern as well as the total amount of label incorporated is dependent on the ratio of the labeled and unlabeled lipid to protein. For example, the extent of labeling of cytochrome *c* oxidase is reduced when the amount of reactive aldehyde is lowered and the amount of DOPC is increased, but the labeling of band VII increases relative to that of bands V and VI. Evidently, the reactivities of at least some of the lysines of VII are greater under these conditions.

Although there have been no previous studies of cytochrome *c* oxidase with amine-specific lipid analogues, the data presented here can be compared to previous work with other labeling reagents. Arylazido derivatives of phospholipids have been used to label the hydrophobic regions of the subunits of beef heart cytochrome *c* oxidase (Bisson et al., 1979; Griffith & Jost, 1979; Prochaska et al., 1980). In general, where the cytochrome *c* oxidase has been solubilized in detergents or reconstituted in lipid bilayers, bands I, III, and VII are the most heavily labeled with the arylazido phospholipids, with minor labeling of some of the other subunits (Prochaska et al., 1980). The benzaldehyde phospholipid labels **6** and **7**, in comparison, show heavy labeling of band VII, some labeling of bands III, V, and VI, and essentially no labeling of bands I, II, and IV (Figure 7).

Also, several water-soluble, lipid-insoluble chemical reagents have been used to label the hydrophilic regions of beef heart cytochrome *c* oxidase (Wikstrom et al., 1981). Reaction with diazobenzene[^{35}S]sulfonate ([^{35}S]DABS) labels bands II–V and VII with minor labeling of bands I and VI, and reaction with *N*-(4-azido-2-nitrophenyl)-2-aminoethane[^{35}S]sulfonate (NAP-[^{35}S]taurine) results in labeling of all of the subunits (Prochaska et al., 1980). Reaction with *N*-ethylmaleimide at pH 7 labels primarily bands III and VI, while additional bands are labeled at higher pH (McGeer et al., 1977). The water-soluble benzaldehyde **5** heavily labels bands IV, V, and VII, with some labeling of other bands except band I. It appears, therefore, that all major subunits of cytochrome *c* oxidase have regions exposed to the aqueous phase. The differences in labeling patterns reflect the differences in chemical specificities of these water-soluble reagents.

It is of interest to relate the labeling results to what is known about the structure of beef heart cytochrome *c* oxidase, although this discussion must at this point be tentative in nature because of the complexity and incomplete knowledge of the system. Subunits I–III, coded on mitochondrial DNA, are the largest and most hydrophobic polypeptides of cytochrome *c* oxidase. The primary structures of subunits I–III have been deduced from DNA sequencing, and the structure of subunit II has also been determined by chemical sequencing methods [see reviews by Buse (1984) and Capaldi et al. (1983)]. There are nine, six, and three lysines present in subunits I, II, and III, respectively (Buse et al., 1982, 1983). Since no significant labeling of subunit I is observed with the benzaldehyde lipid analogues of Figure 1 but labeling does occur with arylazido phospholipids, part of the hydrophobic surface of this large subunit is exposed to the phospholipid bilayer, but no lysines are accessible near the polar head groups of the lipids. The same conclusion applies to subunit II, although this polypeptide appears to be labeled to a lesser extent with the arylazido phospholipids. In contrast, subunit III does have amines available to react with the benzaldehyde head groups of the lipid analogues **3**, **4**, **6**, and **7** but appears to be less reactive than the polypeptides of bands V–VII. There is some uncertainty regarding the protein components IV–VII coded by the nucleus and synthesized on cytoplasmic ribosomes. Depending on the details of the particular gel electrophoresis system, bands IV–VII can be resolved in up to 10 different polypeptides (Buse, 1984; Kadenbach et al., 1983). Subunit IV, the largest of the cytoplasmically synthesized polypeptides, contains a hydrophobic stretch of amino acids flanked by polar amino acids, including lysines. Since band IV did not label significantly with the benzaldehyde lipids, these lysines are probably not directly involved in lipid head-group interactions. Band IV was heavily labeled, however, with the water-soluble benzaldehyde **5**, indicating that there are amino groups exposed to the aqueous environment. Bands V and VI consist of several polypeptides, some or all of which are clearly labeled with both the lipid- and water-soluble benzaldehyde derivatives.

Of special interest here is band VII, since it is the most heavily labeled by the benzaldehyde molecules. Band VII corresponds to the polypeptides of band VIII in Buse's nomenclature (Buse, 1984). There are four to five lysines in each of the three small polypeptides VIIIa, VIIIb, and VIIIc. These polypeptides have the character of integral membrane components; i.e., each contains a hydrophobic sequence consisting of about 20 amino acids that could span the bilayer. Ionic residues, including several lysines, are located at the ends of these hydrophobic stretches, and it has been suggested, for example, that the short cationic C-terminus might be involved in interactions with the phospholipid head groups (Buse & Steffens, 1978). Band VII must contact the other subunits of the protein, but the benzaldehyde labeling patterns shown here provide the first experimental evidence that one or more amino groups are exposed to the phospholipid head-group region of the bilayer. Because of the high reactivity and selectivity of the benzaldehyde labels demonstrated here, it should be possible to identify the points at which some of the hydrophobic stretches of the subunits enter the phospholipid bilayer and to identify regions that could interact specifically with negatively charged lipids.

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SUPPLEMENTARY MATERIAL AVAILABLE

Syntheses of aldehyde reagents **1** and **2** and a single hydrocarbon tail analogue of **1** (5 pages). Ordering information is given on any current masthead page.

Registry No. **1** (free acid), 99281-03-5; **2** (free acid), 99281-04-6; [^{14}C]-**2** (free acid), 99281-28-4; **3**, 99281-05-7; [^{14}C]-**3**, 99281-11-5; **4**, 99281-06-8; [3-^{3}H]-**4**, 99281-16-0; **5**, 99281-07-9; (±)-**6** (free acid), 99281-08-0; (±)-methyl-**6** (free acid), 99281-17-1; (±)-[^{3}H]-**6**, 99281-19-3; [^{3}H]-**7**, 99281-09-1; **8**, 99281-10-4; **9**, 69699-62-3; [^{3}H]-**9**, 99281-15-9; DOPC, 4235-95-4; *p*-$CHOC_6H_4O^-Na^+$, 22666-84-8; $P(O)Cl_3$, 10025-87-3; $CH_3(CH_2)_{15}OH$, 36653-82-4; $(MeO)_2P(O)OC_6H_4CHO$-*p*, 99281-20-6; *p*-HOC_6H_4CHO, 123-08-0; $(MeO)_2P(O)OC_6H_4CHTOH$-*p*, 99281-21-7; $(MeO)_2P(O)OC_6H_4CTO$-*p*, 99281-22-8; *p*-$CHOC_6H_4OP(O)(OH)OCH_2CH(OH)CH_2O_2C(CH_2)_7CH{=}CH(CH_2)_8H$, 99281-23-9; $Cl_2P(O)O(CH_2)_{16}H$, 58527-29-0; $HOCH_2CH(OH)CH_2OP(O)(OH)O(CH_2)_{16}H$, 87764-39-4; $(MeO)_3P$, 121-45-9; $(MeO)_2P(O)OCH_2CH(OEt)_2$, 74685-53-3; $MeOP(O)(OH)OCH_2CH(OEt)_2$ (Na salt), 99281-25-1; MeOP-

(O)(OH)$OCH_2CH(OEt)_2$ (Ag salt), 99298-00-7; $CH_3(CH_2)_{15}I$, 544-77-4; MeOP(O)[$O(CH_2)_{15}CH_3$]$OCH_2CH(OEt)_2$, 99281-26-2; MeOP(O)(OCH_2CHO)$O(CH_2)_{16}H$, 99281-27-3; 15-proxylhexadecanol, 99281-12-6; 15-proxylhexadecanal, 99281-29-5; 15-proxyl[1-^{3}H]hexadecanol, 99281-13-7; 17-proxylstearic acid, 69699-61-2; 17-proxyl[3-^{3}H]stearic acid, 99281-14-8; *rac*-1,2-diolein, 3738-74-7; 1,2-dioleoylglycero-3-methyl-4-(hydroxymethyl)phenyl phosphate, 99281-18-2; 14-proxyl[2-^{3}H]stearic acid, 78387-58-3; 14-proxylstearic acid, 66978-86-7; 1-palmitoyl-2-(14-proxylstearoyl)-*sn*-glycero-3-PC, 78709-94-1; 1-palmitoyl-2-(14-proxylstearoyl)-*sn*-glycero-3-PG (Na salt), 99281-24-0; solketal, 100-79-8; bromoacetaldehyde diethyl acetal, 2032-35-1.

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